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THE PATENT OFFICE
-2 AUG 1993
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The Patent Office

Cardiff Road
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1. Your reference
PA9939

2. Patent application number
(The Patent Office will fill in this part)
9918096.0

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Nycomed Amersham plc
White Lion Road
Amersham
Bucks HP7 9LL. GB

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

GB 07395288001

4. Title of the invention

NMR SPECTROSCOPY METHOD

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

ROLLINS, Anthony John
Nycomed Amersham plc
White Lion Road
Amersham
Bucks HP7 9LL. GB

Patents ADP number *(if you know it)* 07395288001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

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Continuation sheets of this form	0
Description	10
Claim *(s)*	3
Abstract	0
Drawing *(s)*	3 + 3 + 3

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	1
Request for preliminary examination and search *(Patents Form 9/77)*	1
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

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11. I/We request the grant of a patent on the basis of this application.

Signature A.J. Rollins Date 29 July 1999

ROLLINS, Anthony John

12. Name and daytime telephone number of person to contact in the United Kingdom ROLLINS, Anthony John 01494 543090

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NMR SPECTROSCOPY METHOD

This invention is concerned with nuclear magnetic resonance spectroscopy. The technique involves observing the spectrum of a nmr active nuclear species in order to obtain information about the environment in which the species is present. The spectra of nmr active nuclei vary depending on their environment, and this is reported in the literature (PNAS, 93, 12932-6, 1996).

Noble gases having non-zero nuclear spin can be hyperpolarised, i.e. have their polarisation enhanced over the equilibrium polarisation, e.g. by the use of circularly polarised light. Preferred techniques for hyperpolarisation include spin exchange with an optically pumped alkali metal vapour and metastability exchange. Noble gases to which this technique can be applied include helium-3, neon-21, krypton-83, xenon-129 and xenon-131. As described by M S Albert *et al* in US Patent 5,545,396, the technique can be used to prepare hyperpolarised noble gases which can then be administered orally for magnetic resonance imaging of the human body.

It is known that the hyperpolarisation of a noble gas can be transferred by physical contact to another nmr active species. Thus WO 97/37239 describes a method which involves: contacting a sample containing an nmr active nucleus with a hyperpolarised noble gas; scanning the sample using nuclear magnetic resonance spectroscopy, magnetic resonance imaging, or both, in order to detect the nmr active nucleus. It will be understood that magnetic resonance imaging involves determining the presence or location of an nmr active nucleus, and is contrasted with nmr spectroscopy.

Additionally, hyperpolarisation may be imparted to atoms of significance in biological systems, e.g. carbon, nitrogen, phosphorous or even hydrogen by a variety of other techniques. Thus, hyperpolarisation may be directly imparted using a very low temperature suitably below 1°

Kelvin, preferably as close to 0° Kelvin as possible, and a high magnetic field. Alternatively, it may be imparted by dynamic nuclear polarisation in which the material is mixed with the paramagnetic species, for example a transition metal such as chromium or manganese and/or a free radical generator, at a moderate magnetic field and low temperature, e.g. by carrying the conversion out in liquid helium and a magnetic field of about 10 gauss or above. Energy, normally, in the form of microwave radiation, will be provided in the process which will initially excite the paramagnetic species which on its decay to the ground state transfers polarisation to an nmr active nucleus of the target material. A further technique is parahydrogen induced polarisation which involves cooling hydrogen to a low temperature, e.g. 20° Kelvin or less, to give parahydrogen. This parahydrogen is then used to hydrogenate an unsaturated target organic molecule imparting hyperpolarisation to the target molecule.

Other methods for preparing hyperpolarised materials include magnetisation transfer and spin refrigeration.

The present invention provides a method which comprises:

a) using an assay reagent containing at least one nmr active nucleus as defined herein to perform an assay,

b) hyperpolarising the nmr active nucleus,

steps (a) and (b) being performed simultaneously or sequentially in either order, and

c) analysing the assay reagent and/or the assay after the assay reagent has been added by nmr spectroscopy.

The assay can be carried out with the nmr active nucleus in

the assay reagent already hyperpolarised or the assay carried out and the nmr active nucleus then hyperpolarised prior, or at the same time, as the assay/assay reagent is analysed by nmr spectroscopy. Whilst the first arrangement enables real time studies of the assay to be carried out, this is often not necessary and, in these circumstances, the second method is very useful. As hyperpolarisation of the nmr active nucleus will often be carried out at a low temperature, e.g. 20° Kelvin or less, the assay can be started and then effectively frozen by lowering the temperature and, hyperpolarising the assay/assay reagent by nmr spectroscopy. By carrying out this process a number of times, either on the same assay or on parallel assays, a series of "snap-shots" of how the assay is proceeding may be obtained.

When hyperpolarisation is effected by exchange in solution phase, the agent supplying spin can be introduced as a single bolus, as a continuous administration or as an intermittent bolus.

Agents, such as organic solvents, may be added to the assay, and/or to the nmr active nucleus if this is to be hyperpolarised prior to the assay, in order to prolong the stability of the hyperpolarised nmr active nucleus in the assay reagent.

Assays can be carried out by quantifying the appearance of ,the continued presence of, or the disappearance of spectral patterns. For example, on binding or hybridisation of an assay reagent the chemical shift of the signals derived from the nmr active nucleus in the assay reagent will change.

It will be apparent to those skilled in the art that some nmr active nuclei, also referred to herein as hyperpolarisable isotopic atoms, retain their hyperpolarisation for a longer period than others at a given set

of physical parameters, for example carbon 13 has a longer life than nitrogen 15 which has a longer half life than phosphorous 31. Thus, the order in which steps (a) and (b) are carried out may, to some extent, be determined by the choice of nmr active nucleus. Whilst there may be advantages in carrying out the hyperpolarisation of the assay and then monitoring its nmr spectra during the reaction, it is possible to "freeze" the reaction at any time by reducing the temperature after the assay reagent has been added and then hyperpolarising the nmr active nucleus and comparing the spectra obtained with that of the assay reagent in a state where it has not biologically or chemically reacted.

As used herein, nmr active nuclei are those having non-zero nuclear spin and include hydrogen, carbon-13, nitrogen-15, fluorine-19, silicon-29 and phosphorus-31. Of these carbon-13 is preferred. Carbon-13 is present at a natural abundance (relative to carbon-12) of about 1%. Just as the labelling of organic compounds with carbon-14 is widely practised, so organic compounds can be labelled or enriched with carbon-13, either generally or at specific positions in the molecule. Preferably organic compounds for use in this invention contain carbon-13, either generally or at least one specific position, at an abundance of at least 5%, suitably at least 50%, preferably at least 90% and ideally at approaching a 100%. By the method of this invention, carbon-13-labelled compounds can provide all the information provided by corresponding carbon-14-labelled compounds and much more besides. Hydrogen, including its isotopes deuterium and tritium, is also a particularly suitable nmr active nuclei for use in assays of the present invention.

An assay reagent is a substance or compound that takes part in an assay, by being introduced as an initial reagent or by being formed *in situ* and perhaps transiently during the assay, or by being formed as a product of the assay. An assay is a test performed partly or wholly *ex vivo*

in which a physical or chemical change involving a biological species is observed. A biological species is one which is present in living systems or which is introduced into and is reactive with such systems.

Preferably, step c) is performed by examining the assay reagent using both nmr spectroscopy to obtain more than one spectrum, and magnetic resonance imaging to obtain one or more discrete spectral location, and repeating the examination at least once so as to obtain quantitative information about kinetic or time-dependant alteration in chemistry, environment or structure of the assay reagent.

Assays envisaged according to this invention include competition assays, binding assays, immunoassays, hybridisation assays, test involving macro-organisms, and binding studies performed on tissue sections, cultured cells or micro-organisms. Preferred examples are discussed in the following paragraphs. By labelling at specific chemical positions, the chemical and biological properties of the molecule will be unaltered. However, each will have a unique nmr spectrum. Thus dual, triple etc labelling experiments can be carried out and 'stop-flow' measurements be made with identical chemical species. For example, the six carbon atoms in glucose could be individually or collectively replaced by carbon-13, so that one to six of the carbon atoms are carbon-13 which can be hyperpolarised. Each hyperpolarised carbon-13 will give rise to a chemical shift, which will be specific to that individual carbon and differentiable from other carbon-13 positions in the molecule, i.e. C-1 will be different from C-2, etc.

In one aspect, the present invention provides a method which comprises:

a) using a hyperpolarised noble gas to effect nuclear polarisation of an assay reagent comprising at least one nmr active nucleus other than

the noble gas,

b) using the assay reagent to perform an assay,

steps a) and b) being performed simultaneously or sequentially in either order, and

c) analysing by nmr spectroscopy an assay reagent comprising the nmr active nucleus.

The hyperpolarised noble gas is helium-3, neon-21, krypton-83, xenon-129 or xenon-131, preferably helium-3 or xenon-129, as these are now commercially available at high isotope purity with a high degree of hyperpolarisation and an adequate half life.

In step a), a hyperpolarised noble gas is used to effect nuclear polarisation of an assay reagent comprising at least one nmr active nucleus other than the noble gas. This polarisation may be effected by physical contact. The hyperpolarised gas may be in the gas phase, or may alternatively be liquid e.g. by being dissolved in a lipid or fluorocarbon solvent, or a solid e.g. by being absorbed on to a solid surface. The assay reagent may be solid but is generally fluid. A hyperpolarised gas may be bubbled into a fluid assay reagent. Or a hyperpolarised gas solution may be mixed with a fluid assay reagent. The hyperpolarised gas may be cooled and/or maintained in a magnetic field to preserve the hyperpolarisation. Similarly the resulting assay reagent comprising at least one polarised nmr active nucleus may preferably be cooled and/or maintained in a magnetic field in order to preserve the polarisation.

Hybridisation assays are very widely used for sequencing and for detection of point or deletion mutations, When a conventionally labelled polynucleotide probe is hybridised with a polynucleotide target, analysis of the melting temperature or other property of the hybrid can give some limited information about the nucleotide sequence of the target.

According to the present invention, a probe labelled with a polarised nmr active nucleus can give different and more extensive information about the target than has previously been available. This is because a polarised nmr active nucleus generates an nmr spectrum which is dependent on its environment; and that environment extends beyond the labelled molecule itself to other molecules in the immediate vicinity. Thus for example a nucleotide labelled with polarised carbon-13, when incorporated in a single stranded polynucleotide, can give information about two or more adjacent nucleotide residues in the chain. When that labelled polynucleotide probe is hybridised with a polynucleotide target, nmr spectroscopic analysis of the nmr carbon-13 label can give information, not only about the complementary nucleotide residue in the target, but also about two or more adjacent nucleotide residues in the target.

Nucleosides or nucleotides or nucleotide analogues can readily be enriched with carbon-13 at one or several specified points in the molecule. Polarisation of the carbon-13, by contact with a hyperpolarised noble gas, may be effected either before, during or after incorporation of the monomer into a polynucleotide; and before, during or after hybridisation of that polynucleotide with a complementary strand.

Figure 1 demonstrates a hybridisation assay in which the use of an oligonucleotide or polynucleotide to detect the presence of single nucleotide polymorphs (SNPs) in a gene, or fragment of a gene. An oligonucleotide or polynucleotide probe is prepared in which one or more of the atoms has been replaced by a hyperpolarisable isotope, e.g. carbon – 13, nitrogen – 15 or phosphorous – 31. This probe is then hybridised to the gene or the gene fragment. The probe will be "targeted" to information –rich parts of the gene and may be selected so that it binds only to that part of the DNA containing a specific mutation, or, potentially, more than one mutation. If desired, a set of probes, each probe containing a hyperpolarisable isotope, can be added to a gene or gene fragment, each probe being targeted to a different part of the gene/gene

fragment. As each probe will have a characteristic chemical shift by nmr spectroscopy, the spectrum of the mixture of the probes with the target can be taken and resolved to indicate which probes have bound and which not.

The probe may be polarised before, during, or after hybridisation and a determination carried out by nmr of whether a shift has occurred in the signal obtained from the hyperpolarised isotopic atom(s). If a shift has occurred then the probe is in a different chemical environment indicating hybridisation. Clearly information can be obtained from both positive and negative results, e.g. a probe could be constructed from the "natural" gene, and if results indicate that this has failed to bind, probes could be tested containing anticipated mutations. This technique facilitates itself to use of an array-type format in which a number of hyperpolarisable probes are used in the assay which each vary by one nucleotide. The identity of the SNP can be determined by the hybridisation pattern of the probes to the gene/gene fragment.

Further information can be generated by the spin dipole spectra and complex spectral resolution.

Many assays involve a reaction in which a chemical bond is broken. According to an aspect of the present invention, an assay reagent is an organic compound comprising one or more nmr active nuclei associated with a bond which is broken during the course of the assay. In the case of a single nmr active nucleus this is located preferably at the actual site of the breaking of the chemical bond such that the change in local environment of the active nucleus subsequent to the bond breaking will give rise to a change in the spectrum of the nmr active nucleus. The nmr spectra of two or more active nuclei will be different, depending on whether they are present within the same molecule or in different molecules. When two or more active nmr nuclei are in an appropriate proximity to one another they are said to be spin coupled. This gives rise to a distinct nmr spectrum which can be monitored. It is therefore possible

to analyse by nmr spectroscopy the rate and extent of the bond's breaking by the disruption of the spin coupling. In this and other assays, the assay reagent may be analysed repeatedly by nmr spectroscopy so as to generate information about a change with time of the assay reagent.

Figure 2 demonstrates a proteolysis assay. The starting substrate for the reaction contains two hyperpolarisable isotopes, in this case carbon13, which are sufficiently close together, either by virtue of being reasonably adjacent in the chain of amino acids comprising the molecule, or by the 3-dimensional conformation of the molecule bringing them together, so that in the nmr spectra spin-spin coupling of the signal results. The nmr spectra of the molecule is recorded.

The molecule is then brought into contact with an enzyme capable of altering the chemical composition of the substrate. If cleavage occurs between the amino acids containing the hyperpolarisable isotopic atoms, the spin-spin coupling observed in the nmr spectra disappears and two new spectra are apparent, one for each of the individual hyperpolarisable isotopes. if there is no cleavage, the original spectra remains.

A similar assay can be carried out where the starting substrate is a chain of nucleotides and the cleavage enzyme an endonuclease.

In another aspect of the invention, an assay reagent may be administered to a macro-organism, e.g. a human or animal, and nmr spectroscopic analysis performed of excreta, ~~e~~.g. urine, faeces or breath, of the macro-organism. In this case, the assay reagent may be labelled by contact with a hyperpolarised gas before administration; or alternatively the excreta may be labelled before spectroscopic analysis.

In yet another aspect of the invention, an assay reagent may be used in binding studies on bacteria or other eukaryotic or prokaryotic micro-organisms or cultured cells.

Assays according to this invention may conveniently be carried out in multiwell plates. An assay reagent in each well may be labelled by

contact with a hyperpolarised noble gas, prior to addition of other assay reagents. Or an assay reagent in bulk may be labelled by contact with a hyperpolarised noble gas prior to being dispensed into individual wells of a multiwell plate. In many cases, assays can be performed in a homogenous mode, that is to say without the need for a separation step to remove one fraction of the labelled reagent.

In addition in cases where the spectra of the ^{13}C labelled assay components are distinct from one another more than one assay may be performed and simultaneously monitored in a single well or spot of a multi-assay array. This would allow multiplexing of several related or unrelated assays in parallel within a single well or spot in a multi-assay array which is either ordered or random. In addition the technique may be applied to aerosol droplets where no well, container or surface is used to contain the assay and to analysis of samples in flow-through devices.

Figure 3 illustrates how the incorporation of a material (for example an amino acid) into a cell can be measured. The material incorporates a hyperpolarisable isotopic atom, in this case carbon13. Its nmr spectra in a hyperpolarised state in the media used for the experiment is recorded. If the material crosses the cell membrane then the environment in which the material finds itself will change and this will effect the nmr chemical shift of the material. The precise chemical shift will depend on the environment of the material within the cell, for example it may be possible to identify whether it has crossed into the cell nucleus. Alternatively, the material may be bound to the surface of the cell, again a different spectra will result. In addition, metabolites that contain a hyperpolarisable isotopic atom may be detected either inside the cell of after they are excreted from this. the spectra obtainable on these metabolites can be used for their identification and/or to give information on their structure.

CLAIMS

1. A method which comprises:

a) using an assay reagent containing at least one nmr active nucleus as defined herein to perform an assay, and

b) hyperpolarising the nmr active nucleus; steps (a) and (b) being performed simultaneously or sequentially in either order, and

c) analysing the assay reagent and/or the assay after the assay reagent has been added by nmr spectroscopy.

2. A method which comprises:

d) using a hyperpolarised noble gas to effect nuclear polarisation of an assay reagent comprising at least one nmr active nucleus other than the noble gas,

e) using the assay reagent to perform an assay,

steps a) and b) being performed simultaneously or sequentially in either order, and

f) analysing by nmr spectroscopy an assay reagent comprising the nmr active nucleus.

3. The method of claim 1 or 2, wherein step c) is performed by examining the assay reagent using both nmr spectroscopy to obtain more than one spectrum, and magnetic resonance imaging to obtain one or more discrete spectral location, and repeating the examination at least once so as to obtain quantitative information about kinetic or time-dependant alteration in chemistry, environment or structure of the assay reagent.

4. The method of any claims to 3, wherein the nmr active nucleus is ^{15}N, ^{19}F, ^{31}P or ^{13}C.

5. The method of any one of claims to 3, wherein the nmr active nucleus is hydrogen, deuterium or tritium.

6. The method of claim 4 or 5, wherein the assay reagent is a compound of which at least one atom contains an artificially high concentration of the nmr active nucleus.

7. The method of any one of claims 1 to 5, wherein the assay reagent is a nucleotide, or nucleotide analogue, polynucleotide, amino acid, amino acid analogue, polypeptide or protein.

8. The method of any one of claims 1 to 7, wherein the assay is a nucleic acid hybridisation assay.

9. The method of any one of claims 1 to 7, wherein the assay is a binding assay.

10. The method of any one of claims 1 to 9, wherein the assay reagent is an organic compound comprising one or more nmr active nuclei associated with a bond which is broken during the course of the assay.

11. The method of claim 10, wherein the assay reagent contains two or more nmr active nuclei present in a spin coupled state and giving rise to distinct spectra, an assay performed resulting in a change in chemical structural or environmental status of the assay reagent, and the changes are monitored.

12. The method of any one of claims 1 to 11, wherein the assay reagent is analysed repeatedly in step c) so as to generate information about a change with time of the assay reagent.

13. The method of any one of claims 1 to 12, wherein an organic compound specifically labelled with an nmr active nucleus is administered to a micro-organism, macro-organism or cultured cells and an excretion product of the labelled compound is analysed by nuclear magnetic resonance spectroscopy, nuclear magnetic resonance imaging or both.

14. The method of any one of claims 1 to 12, wherein the assay is a binding study performed using micro-organisms or cultured cells.

15. The method of any one of claims 1 to 12, wherein the assay is performed in a homogenous mode.

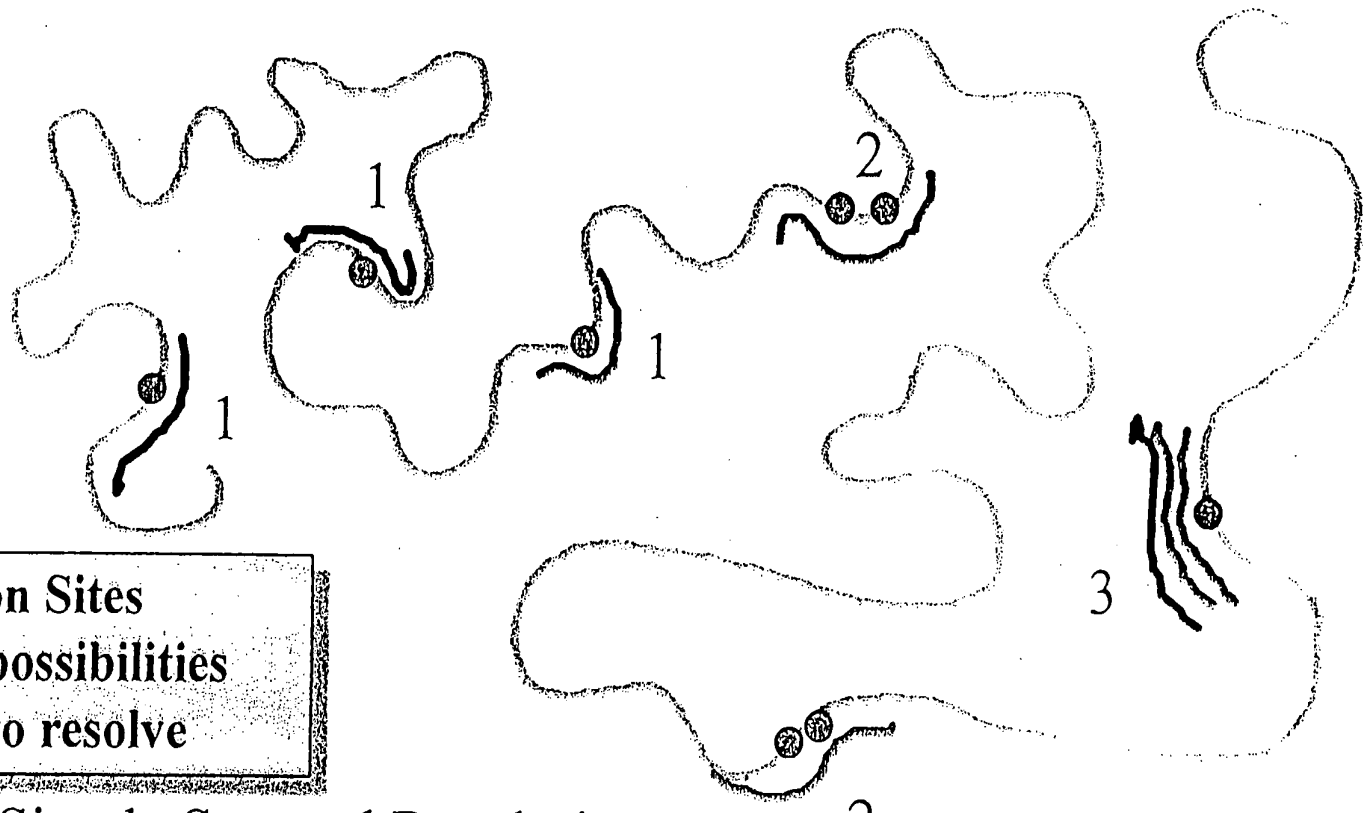
16. The method of any one of claims 1 to 13, wherein more than one assay is multiplexed and monitored by nmr spectroscopy and optionally magnetic resonance imaging.

17. The method of any one of claim 1 to 16, wherein the assay is performed in a multiwell or multispot assay array.

18. The method of any one of claim 1 to 16, wherein step c) is performed in an aerosol or flow-through device applied to aerosol droplets where the well, surface or container is used to contain the assay reagent.

Figure (1)

Multiple SNP detection.



8 Mutation Sites
10 Mutation possibilities
20 Spectra to resolve

1 Simple Spectral Resolution
2 Spin Dipole Spectra
3 Complex Spectral Resolution

Figure (2)

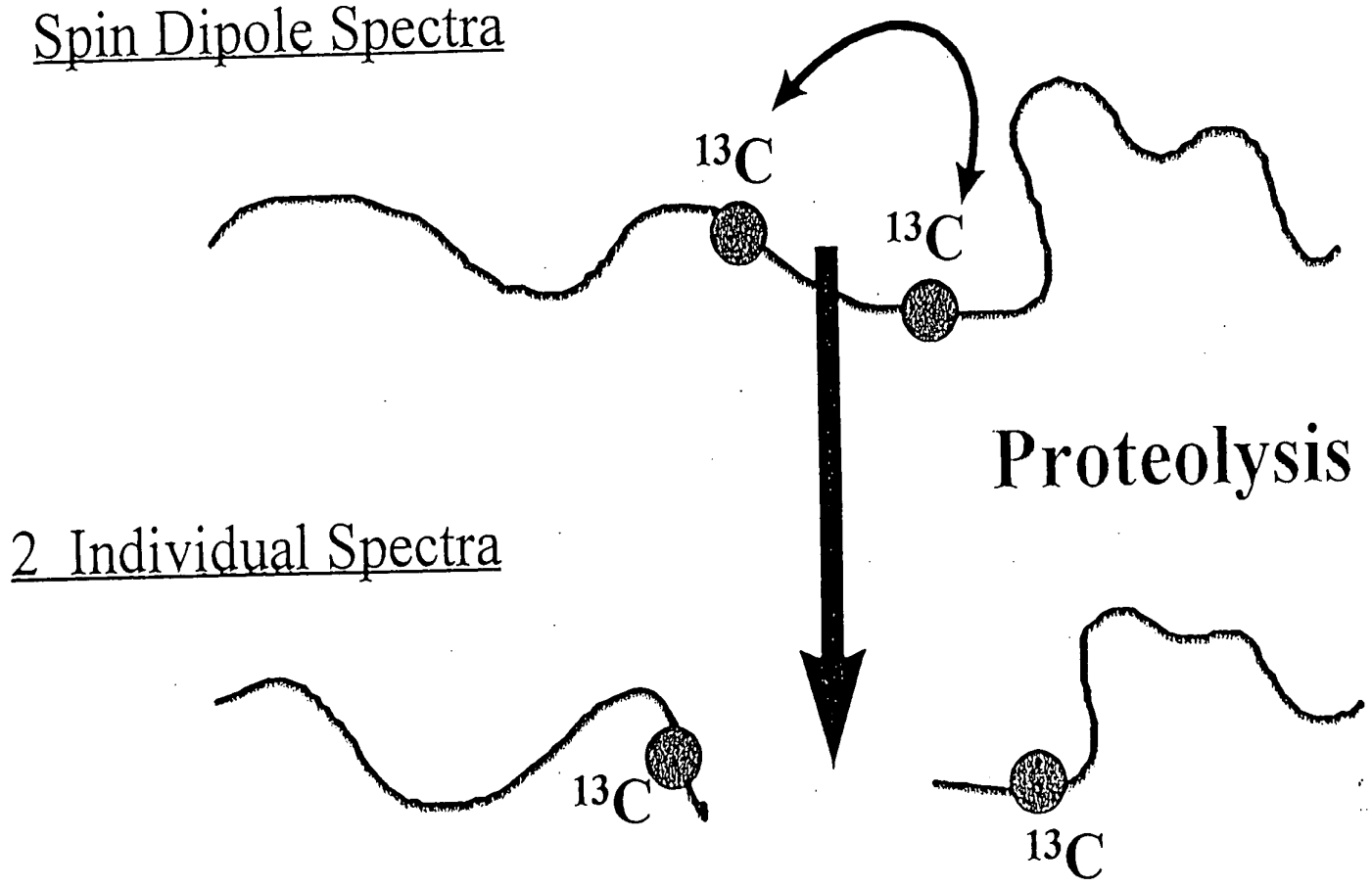
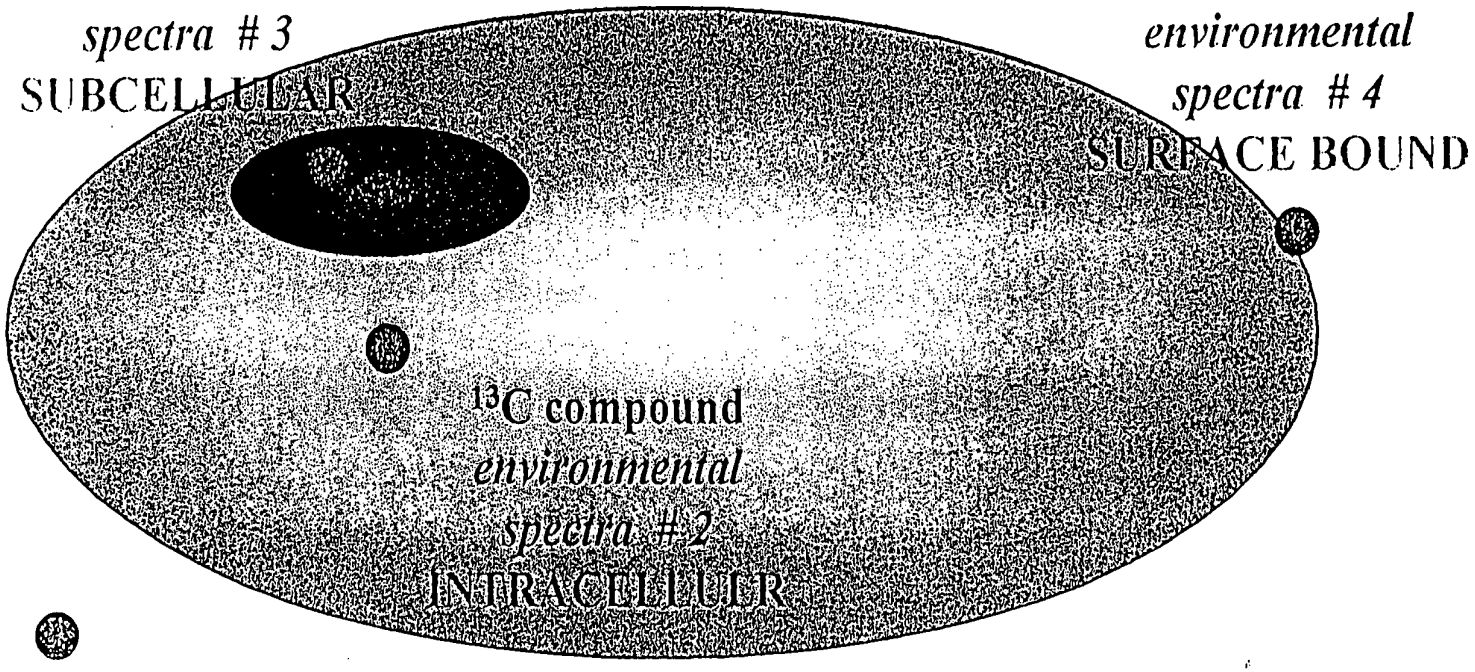

Spin Dipole Spectra
13C
13C
Proteolysis
2 Individual Spectra
13C
13C

Figure (3)


^{13}C compound
environmental
spectra # 3
SUBCELLULAR
^{13}C compound
environmental
spectra # 4
SURFACE BOUND
^{13}C compound
environmental
spectra #2
INTRACELLULR
^{13}C compound
original spectra #1
EXTRACELLULR

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